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Neomyrmecridium asymmetricum sp. nov. from Ecuador

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Abstract—A new species *Neomyrmecridium asymmetricum,* found on decaying leaves of *Theobroma cacao*, is distinguished by grouped conidiophores and polyblastic production of narrow clavate to subclavate, 1-septate, asymmetrical, and yellowish or subhyaline conidia. An ITS- and LSU-based phylogenetic analysis, description, and illustrations are provided. A key and illustrations to *Neomyrmecridium* species are also presented.

Key words—asexual fungi, *Myrmecridiaceae,* taxonomy, tropics

Introduction

Crous & al. (2018a) introduced *Neomyrmecridium* Crous for three species: *N. septatum* Crous (type species), *N. asiaticum* Crous, and *N. sorbicola* (Crous & R.K. Schumach.) Crous. *Neomyrmecridium* is distinguished by macronematous, unbranched, subcylindrical, multiseptate, smooth, brown conidiophores with polyblastic, terminal, subcylindrical, denticulate, pale brown conidiogenous cells. The conidia are solitary, fusoid-ellipsoid, obovoid, hyaline or subhyaline (becoming pale brown with age), septate, smooth, and sometimes encased in mucoid tunica (Crous & al. 2018a). The diversity of microfungi in the Ecuadorian rainforests has received little attention, especially in cacao plantations. During a survey of hyphomycetes associated with plant litter in the Balao cacao plantation, Guayas province, Ecuador (Fig. 1), we collected a *Neomyrmecridium* specimen that differs remarkably from all previously described taxa (Crous & al. 2018a) and for which we propose a new species.

Materials & methods

Collections

Samples of decaying plant materials were collected and placed in plastic bags for transport to the laboratory, where they were washed, treated according to Castañeda-Ruiz & al. (2016), and placed in moist chambers. Pure cultures were obtained by transferring single conidium using a flamed needle to solidified media (with pH adjusted to 6.3) containing corn meal extract mixed 1:1 with carrot extract plus 15 g agar (CMC) or V8 according to Crous & al. (2009). Plates were incubated at 25 °C. Color notations in parentheses are from Kornerup & Wanscher (1984). Mounts were prepared in PVL (polyvinyl alcohol, lactic acid) and measurements made at 1000× magnification. Microphotographs were obtained with an Olympus BX51 microscope equipped with bright field and Nomarski interference optics. The type specimen was deposited in the Herbarium of Universidade Federal de Pernambuco, Recife, Brazil (URM) and cultures obtained from the type specimen were deposited in the Culture collections of Microorganism CIBE (CCM-CIBE), Guayaquil, Ecuador.

DNA extraction, sequencing, and phylogenetic analysis

Isolates CCMCIBE-H304 and CCMCIBE-H304-A were cultured on PDA in darkness for 7 days at 25 °C. DNA was extracted using a modified protocol from Cenis (1992). The primers ITS1/ITS4 were used to amplify ITS regions, including the 5.8S gene (Manter & Vivanco 2007), and LROR/LR5 to amplify the D1/D3 domain of the LSU nrDNA (White & al. 1990). PCR products were sent to Macrogen Inc. (South Korea) for purification and sequencing. Consensus sequences assembled and edited using Geneious (ver. 10.1.2) were later compared with those of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

Each data set was aligned in MEGA 6.0 (Tamura & al. 2013) using ClustalW (Thompson & al. 1994) and refined with MUSCLE (Edgar 2004). The alignment included our strain sequences and those from different genera in the *Myrmecridiaceae* obtained from NCBI (Table 1). ITS- and LSU-based

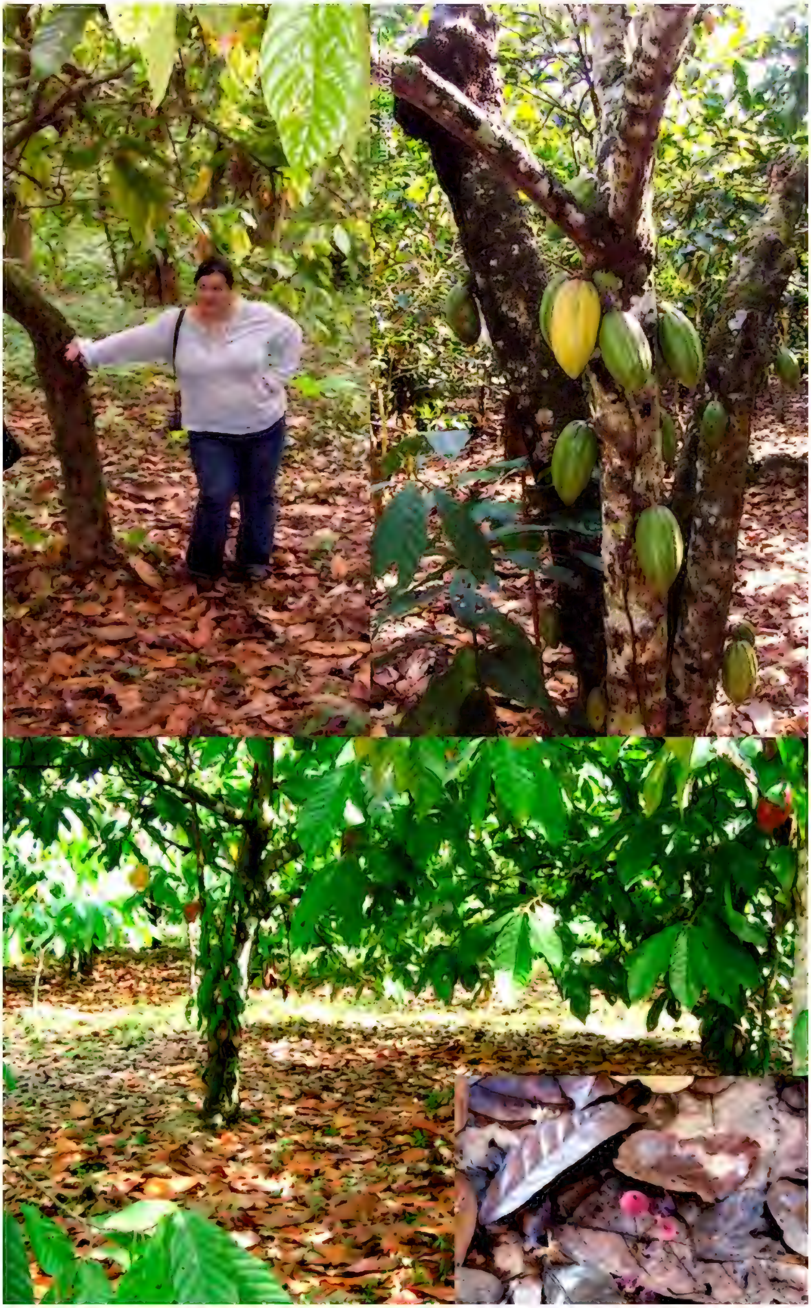

FIG. 1. Balao cacao plantation, Guayas province, Ecuador.

TABLE 1. Sequences of *Neomyrmecridium* and allied taxa used for phylogenetic analyses.

SPECIES	STRAIN	ITS	LSU	REFERENCE
Neomyrmecridium asiaticum	CBS 145080	MK047444.1	MK047494.1	Crous & al. (2018a)
N. asymmetricum	CCMCIBE-H304	MN014057	MN014055	**This study**
	CCMCIBE-H304-A	MN014058	MN014056	**This study**
N. septatum	CBS145073	MK047442.1	MK047492.1	Crous & al. (2018a)
N. sorbicola	CBS:143433	MH107901.1	MH107948.1	Crous & al. (2018b)
Beltraniella endiandrae	CBS 137976	KJ869128	NG_058665.1	Crous & al. (2014)
B. humicola	CBS 203.64	MH858416.1	MH870044.1	Vu & al. (2019
B. portoricensis	NFCCI 3993	KX519516	KX519522.1	Rajeshkumar & al. (2016)
	CBS 856.70	MH859981.1	MH871777.1	Vu & al. (2019)
Cancellidium applanatum	CBS 137655	—	KF833359.1	Zelski & al. (2014)
	CBS 337.76	NR_159777.1	MH872755.1	Vu & al. (2019)
Castanediella acaciae	CPC 24869	KR476728.1	KR476763.1	Crous & al. (2015a)
Cas. cagnizarii	CBS 101043	KP859051.1	KP858988.1	Hernández-Restrepo & al. (2016a)
	CBS 579.71	MH860269.1	MH872031.1	Vu & al. (2019)
Cas. eucalypti	CBS 139897	—	MH878665.1	Vu & al. (2019)
	CBS 139897	NR_137981.1	KR476723.1	Crous & al. (2015a)
Cas. malaysiana	CPC 24918	NR_154810.1	KX306781.1	Hernández-Restrepo & al. (2016b)
Cas. tereticornis	CBS:145068	MK047417.1	MK047468.1	Crous & al. (2018a)
Lasiosphaeria sorbina	GJS L555	AY587934.1	AY436415.1	Miller & Huhndorf. (2004)
Myrmecridium banksiae	CBS 132536	NR_111762.1	NG_042684.1	Crous & al. (2012)
M. flexuosum	CBS 398.76	EU041768.1	EU041825.1	Arzanlou & al. (2007)
M. fluviae	CNUFC-YR61-2	KX839679.1	KX839676.1	Tibpromma & al. (2017)
M. montsegurinum	PRM 934684	KT991674.1	KT991664.1	Réblová & al. (2016)
M. phragmitis	CBS 131311	NR_137782.1	NG_057948.1	Crous & al. (2011)
M. schulzeri	CBS 100.54	EU041769.1	EU041826.1	Arzanlou & al. (2007)
M. spartii	CPC 24953	KR611884.1	KR611902.1	Crous & al. (2015b)

Neopyricularia commelinicola	CBS 128303	—	KM009151.1	Crous & al. (2016a)
	CBS 128303	KM484868.1	KM484982	Klaubauf & al. (2014)
Pararamichloridium caricicola	CBS:145069	MK047438.1	MK047488.1	Crous & al. (2018a)
P. livistonae	CBS 143166	NR_156652.1	NG_058504.1	Crous & al. (2017a)
P. verrucosum	CBS 128.86	MG386030.1	NG_057768.1	Crous & al. (2017a)
Porobeltraniella porosa	NFCCI 3996	KX519520.1	KX519526.1	Rajeshkumar & al. (2016)
Pseudopyricularia bothriochloae	CPC 21650	NR_137838.1	NG_058051.1	Crous & al. (2013)
Ps. hagahagae	CPC 25635	KT950851.1	NG_059616.1	Crous & al. (2015c)
Ps. hyrcaniana	Ck3	NR_158928.1	KY457267.1	Pordel & al. (2017)
Ps. iraniana	IRAN 2761C	NR_158928.1	NG_060183.1	Pordel & al. (2017)
Pyricularia urashimae	CPC 29414	NR_154361.1	NG_059752.1	Crous & al. (2016b)
Pyriculariomyces asari	CPC 27444	—	MH878225.1	Vu & al. (2019)
	CPC 27444	KX228291.1	KX228342	Crous & al. (2013)
Saccharata proteae	CBS:119218	EU552145.1	EU552145.1	Crous & al. (2017b)
Thozetella fabacearum	MFLU 16-1021	KY212754.1	NG_059767.1	Perera & al. (2016)
T. nivea	MUCL 41041	EU825201.1	EU825200.1	Jeewon & al. (2009)
T. pinicola	RJ-2008	EU825197.1	EU825195.1	Jeewon & al. (2009)
T. tocklaiensis	CBS 378.58	MH857817.1	MH869349.1	Vu & al. (2019)
Vermiculariopsiella acaciae	CPC 26291	KX228263.1	KX228314.1	Crous & al. (2013)
V. dichapetali	CBS:143440	MH107924.1	MH107970.1	Crous & al. (2018b)
V. eucalypti	CPC 25525	KX228251.1	KX228303.1	Crous & al. (2013)
V. eucalypticola	CBS:143442	MG386070.1	MG386123.1	Crous & al. (2017a)
V. immersa	MUCL39135	KJ476965	KJ476961.1	Becerra-Hernández & al. (2016)
V. lauracearum	CBS:145055	MK047436.1	MK047487.1	Crous & al. (2018a)
V. pediculata	CBS132484	MH866028.1	MH877476.1	Vu & al. (2019)
Woswasia atropurpurea	WC 18S	NR_154480.1	JX233658.1	Jaklitsch & al. (2013).
Xylochrysis lucida	CBS 135996	—	MH877601.1	Vu & al. (2019)
	CBS 135996	NR_132063.1	NG_058028.1	Réblová & al. (2014)

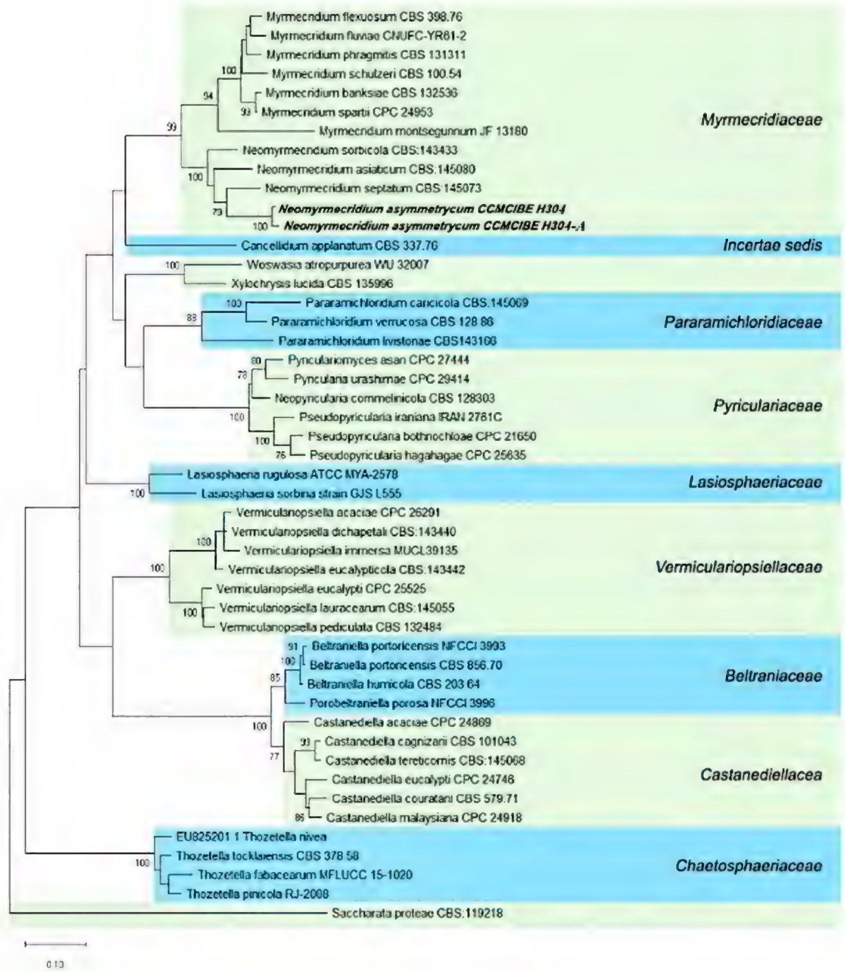
Myrmecridium flexuosum CBS 398.76
Myrmecridium fluviae CNUFC-YR61-2
Myrmecridium phragmitis CBS 131311
Myrmecridium schulzeri CBS 100.54
Myrmecridium banksiae CBS 132536
Myrmecridium spartii CPC 24953
Myrmecridium montsegurinum JF 13180
Neomyrmecridium sorbicola CBS:143433
Neomyrmecridium asiaticum CBS:145080
Neomyrmecridium septatum CBS 145073
Neomyrmecridium asymmetrycum CCMCIBE H304
Neomyrmecridium asymmetrycum CCMCIBE H304-A
Myrmecridiaceae
Cancellidium applanatum CBS 337.76
Incertae sedis
Woswasia atropurpurea WU 32007
Xylochrysis lucida CBS 135996
Pararamichloridium caricicola CBS:145069
Pararamichloridium verrucosa CBS 128.86
Pararamichloridium livistonae CBS143166
Pararamichloridiaceae
Pyriculariomyces asari CPC 27444
Pyricularia urashimae CPC 29414
Neopyricularia commelinicola CBS 128303
Pseudopyricularia iraniana IRAN 2781C
Pseudopyricularia bothriochloae CPC 21650
Pseudopyricularia hagahagae CPC 25635
Pyriculariaceae
Lasiosphaeria rugulosa ATCC MYA-2578
Lasiosphaeria sorbina strain GJS L555
Lasiosphaeriaceae
Vermiculariopsiella acaciae CPC 26291
Vermiculariopsiella dichapetali CBS:143440
Vermiculariopsiella immersa MUCL39135
Vermiculariopsiella eucalypticola CBS:143442
Vermiculariopsiella eucalypti CPC 25525
Vermiculariopsiella lauracearum CBS:145055
Vermiculariopsiella pediculata CBS 132484
Vermiculariopsiellaceae
Beltraniella portoricensis NFCCI 3993
Beltraniella portoricensis CBS 856.70
Beltraniella humicola CBS 203.64
Porobeltraniella porosa NFCCI 3996
Beltraniaceae
Castanediella acaciae CPC 24869
Castanediella cagnizarii CBS 101043
Castanediella tereticornis CBS:145068
Castanediella eucalypti CPC 24746
Castanediella couratarii CBS 579.71
Castanediella malaysiana CPC 24918
Castanediellacea
EU825201.1 Thozetella nivea
Thozetella tocklaiensis CBS 378.58
Thozetella fabacearum MFLUCC 15-1020
Thozetella pinicola RJ-2008
Chaetosphaeriaceae
Saccharata proteae CBS:119218
100
94
93
99
100
79
100
100
100
88
80
78
100
100
75
100
100
100
100
91
100
85
100
93
77
86
100
0.10

Fig. 2. The tree derived from the phylogenetic analysis using concatenated sequences of the LSU and ITS of *Myrmecridiales* revealed that *Neomyrmecridium asymmetricum* and *N. septatum* CBS:145073 were nested in a well-supported subclade (bootstrap value of 79).

phylogenies were generated using Maximum Likelihood (ML) with the best nucleotide substitution model in MEGA 6.0 (Tamura & al. 2013).

Best models used were (for LSU) Tamura-Nei with Gamma distribution and (for ITS) Kimura 2-parameter with Gamma distribution and Invariant sites (G+I). The best nucleotide substitution model for the combined LSU + ITS analysis was the General Time Reversible with Gamma distribution and Invariant sites (G+I). Bootstrap analysis of 1,000 replicates was used to assess the reliability of the reconstructed phylogenies. ML bootstrap values ≥70% were considered significant. DNA sequences generated in this study were deposited in GenBank (Table 1).

Phylogeny

The BLAST query revealed that the CIBE H304 and CIBE H304-A LSU sequences of *N. asymmetricum* showed a 97% similarity with *N. septatum* CBS145073 and *N. asiaticum* CBS145080. However, they showed sequence identity of 94–96% with LSU sequences of *Myrmecridium* species, also belonging to the *Myrmecridiaceae*. The CIBE H304 and CIBE H304-A ITS sequences showed a 90 % similarity with *N. sorbicola* CBS143433, the highest value matched after blast search.

We carried out individual and combined analyses of the LSU and ITS loci to assess relationships with members of the *Myrmecridiales* (*Sordariomycetes*) (Fig. 2). The final concatenated analysis encompassed 48 sequences and comprised 1118 bp (ITS 555 bp, LSU 563 bp). The ML tree nested both *N. asymmetricum* isolates (CIBE H304 and CIBE H304-A) and *N. septatum* CBS145073 in a well-supported subclade (bs = 79%), with *N. asiaticum* CBS 145080 as the closest sister species (Fig. 2).

Taxonomy

Neomyrmecridium asymmetricum R.F. Castañeda, Serrano & D. Sosa, **sp. nov.** Figs 3–5

MycoBank MB 831330

Differs from *Neomyrmecridium sorbicola* by its narrow clavate to subclavate, 1-septate, asymmetrical conidia.

Type: Ecuador, Guayas Province, Guayaquil, Balao, 2°48 S 79°40 W, on decaying leaves of *Theobroma cacao* L. (*Malvaceae*), 8 July 2017, F. Espinoza & S. Pérez-Martínez (**Holotype,** URM 90896; ex-type cultures, CCMCIBE-H304, GenBank MN014057, MN014055; and CCMCIBE-H304-A, GenBank MN014058, MN014056).

Etymology: *asymmetricum*- (Latin), meaning asymmetric.

Colonies on the natural substrate hairy, effuse, amphigenous, yellowish brown. Mycelium superficial and immersed, composed of branched, 1–2.5 µm diam, smooth, brown hyphae. Conidiophores macronematous, mononematous, grouped, slightly fasciculate, erect, straight, cylindrical, unbranched, 3–15-septate, brown, pale brown toward the apex, 40–210 × 3.5–9 µm, smooth. Conidiogenous cells polyblastic, terminal, integrated, cylindrical or subcylindrical, indeterminate, with several sympodial extensions, denticulate, with tiny cylindrical denticles, pale brown, 8–35 ×

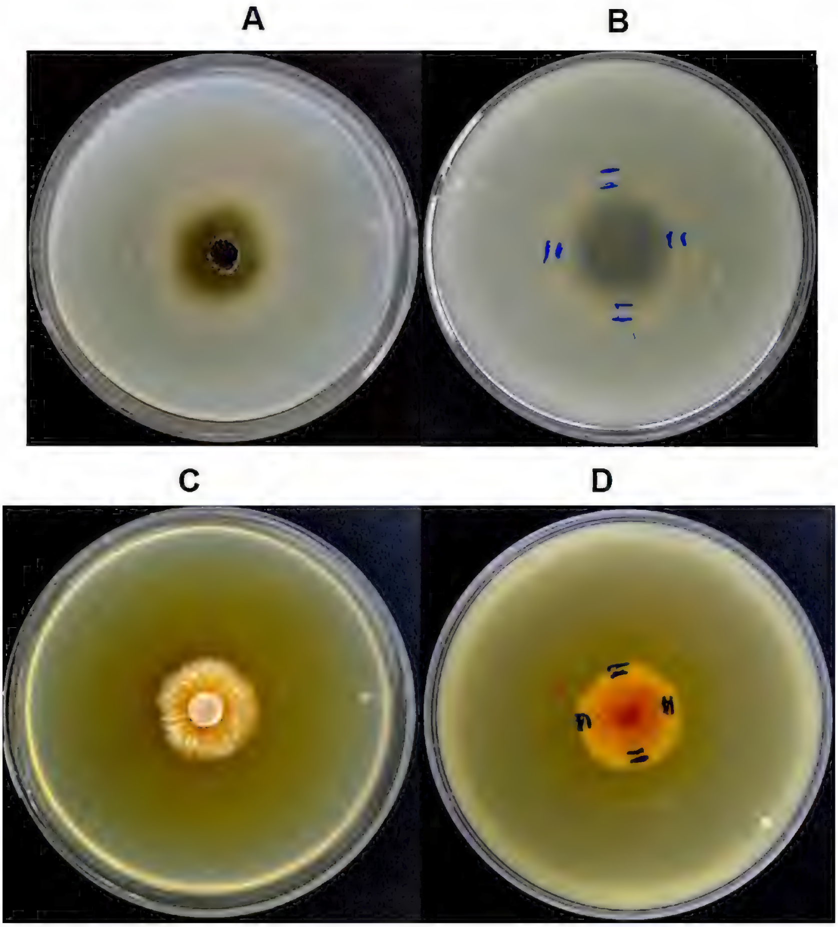

Fig. 3. *Neomyrmecridium asymmetricum* (cultures ex holotype, URM 90896). A–D. Colonies and reverses on CMC (A–B) and V8 agar (C–D).

3.5–5 µm. Conidial secession schizolytic. Conidia solitary, acropleurogenous, narrow clavate or subclavate, 1-euseptate, asymmetrical, 12–15 × 2–3 µm, basal cell 7–10 µm long, apical cell 4–6 µm long, yellowish or subhyaline, smooth-walled.

Culture characteristics: Colonies on CMC attaining 22 mm in 7 days at 25°C, flat, subfelted, olive (3/E5). Mycelium mostly immersed, scarcely aerial toward the entire margin. Reverse dark green (30/F6). Hyphae septate,

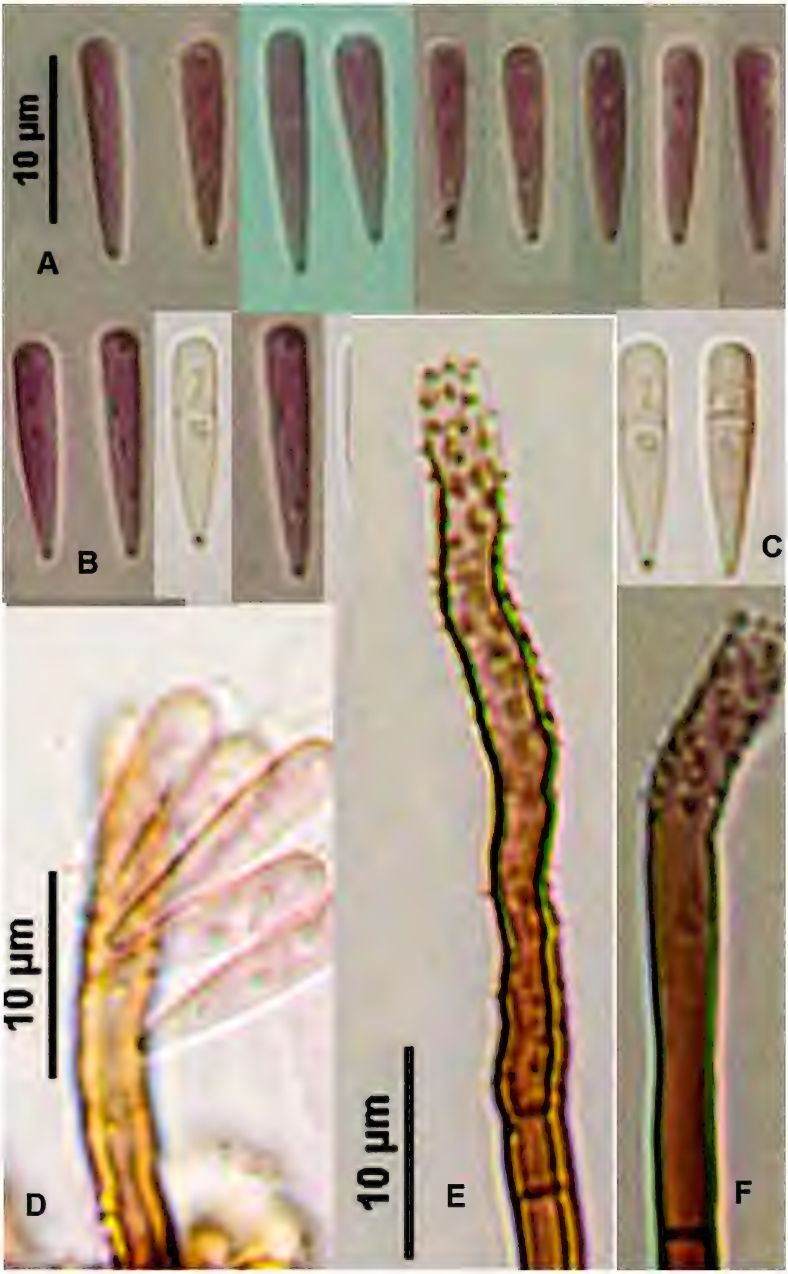

Fig. 4. *Neomyrmecridium asymmetricum* (holotype, URM 90896).
A–C. Conidiophores and conidiogenous cells. D. Conidiogenous cell.

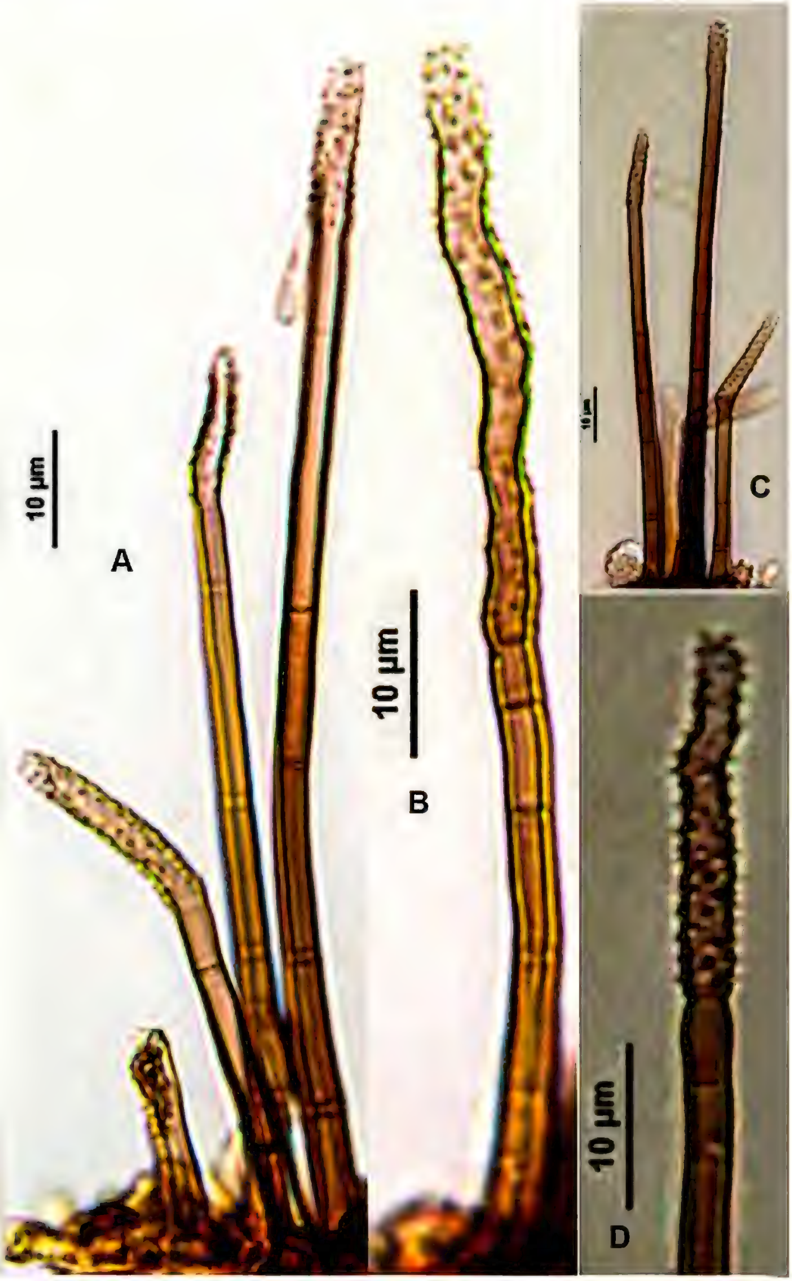

Fig. 5. *Neomyrmecridium asymmetricum* (holotype, URM 90896).
A–C. Conidia. D. Conidiogenous cell and conidia. E–F. Conidiogenous cells.

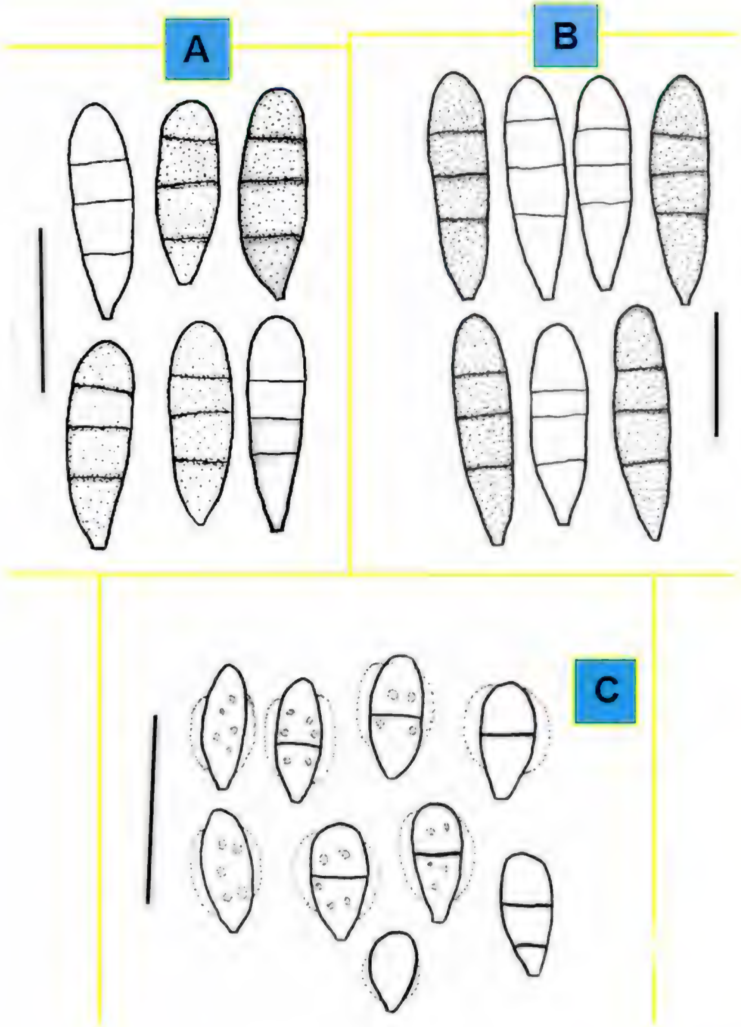

Fig. 6. Representative conidia of *Neomyrmecridium* spp. (re-drawn from the literature). A. *N. asiaticum* (Crous & al. 2018a). B. *N. septatum* (Crous & al. 2018a). C. *N. sorbicola* (Crous & al. 2018b). Scale bars = 10 µm.

subhyaline to pale olivaceous-brown, smooth, 1.5–2 µm diam. Sporulation occurred after 4 days, producing conidia similar to those observed from nature. Colonies on V8 agar attaining 12 mm in 7 days at 25°C, flat concentric near cottony center, filamentous-filiform toward margin, yellowish orange (4/A7).

Reverse deep orange (5/A8) at the center, light orange (5/A6) toward the margin. Sporulation poor and sparsely after 5 days, conidia similar to those observed from nature.

NOTE: *Neomyrmecridium sorbicola* (Crous & al. 2018a,b) is superficially similar to *N. asymmetricum* in producing 1-septate conidia, but *N. sorbicola* differs in its pale brown obovoid conidia with median regions surrounded by a mucoid tunica (FIG. 6). *Neomyrmecridium septatum* is separated by its conidia, which are fusoid-ellipsoid, mostly 3-septate, guttulate, pale brown, and with a mucoid tunica encasing the upper two thirds (Crous & al. 2018a) (FIG. 6).

Morphological and phylogenetic analyses support *N. asymmetricum* as a new species in *Myrmecridiaceae*.

Key to *Neomyrmecridium* species

1. Conidia mostly 1-septate 2
1. Conidia mostly more than 1-septate 3
2. Conidia clavate or subclavate, 12–15 × 2–3 µm, asymmetrical,
    basal cell 7–10 µm long, apical cell 4–6 µm long,
    yellowish or subhyaline .... ***N. asymmetricum***
2. Conidia obovoid, (7–)8–10(–15) × 4(–5) µm, initially hyaline
    (pale brown in age), (0–)1(–3)-septate, with a 1–2 µm thick
    mucoid tunica surrounding the medial region .... ***N. sorbicola***
3. Conidia fusoid-ellipsoid, (12–)14–16(–20) × (3.5–)4(–5) µm, hyaline
    (pale brown in age), (1–)3-septate, guttulate, with a 1–2 µm thick
    mucoid tunica encasing the upper two thirds .... ***N. septatum***
3. Conidia ellipsoid to obovoid, (13–)15–17 × 4–5 µm, pale brown,
    (2–)3-septate, guttulate, surrounded by a 0.5 µm thick
    gelatinous tunica .... ***N. asiaticum***

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